

Purification and Characterization of Jerdonitin, a Non-hemorrhagic Metalloproteinase from *Trimeresurus jerdonii* Venom

CHEN Run-qiang^{1,2}, JIN Yang¹, WU Jian-bo^{1,2}, ZHONG Shu-rong^{1,2}, ZHU Shao-wen¹,
LU Qiu-min¹, WANG Wan-yu¹, XIONG Yu-liang^{1,*}

(1. Department of Animal Toxinology, Kunming Institute of Zoology, the Chinese Academy of Sciences, Kunming 650223, China;

2. Graduate School of the Chinese Academy of Sciences, Beijing 100039, China)

Abstract: Previously, we have purified Jerdonitin from *Trimeresurus jerdonii* venom. Compared with other P-II class snake venom metalloproteinases (SVMPs), Jerdonitin has a primary structure comprising metalloproteinase and disintegrin domains. However, no hemorrhagic and fibrinogenolytic activities were detected for Jerdonitin. We thought that organic buffer of high performance liquid chromatography (HPLC) might affect its enzymatic activity. In this study, we purified Jerdonitin by another procedure excluding the HPLC. It was homogenous as judged by SDS-PAGE and had an apparent molecular weight of 36 kDa under non-reducing conditions and 38 kDa under reducing conditions, respectively. Like other typical metalloproteinases, Jerdonitin preferentially degraded alpha-chain of human fibrinogen and this fibrinogenolytic activity was completely inhibited by EDTA, but not by PMSF. It was interesting that Jerdonitin did not induce hemorrhage after intradermal injection in mice.

Key words: *Trimeresurus jerdonii*; SVMP; Hemorrhage; Fibrinogenolytic activity

菜花烙铁头蛇毒金属蛋白酶 Jerdonitin 的分离纯化和理化性质

陈润强^{1,2}, 金 扬¹, 吴健波^{1,2}, 钟树荣^{1,2}, 朱绍文¹,
吕秋敏¹, 王婉瑜¹, 熊郁良^{1,*}

(1. 中国科学院昆明动物研究所 动物毒素研究室, 云南 昆明 650223; 2. 中国科学院研究生院, 北京 100039)

摘要: 以前从菜花烙铁头蛇毒中分离纯化到 Jerdonitin。与其他 II 型蛇毒金属蛋白酶相比, Jerdonitin 由金属蛋白酶和去整合素两个结构域组成。但没有检测到其出血和纤维蛋白原降解活性, 推测可能高压液相色谱的有机溶液影响了其酶活性。采用不含高压液相色谱柱层析的新分离手段分离得到 Jerdonitin。Jerdonitin 在还原和非还原 SDS-PAGE 电泳中分别呈现一条表观分子量为 38 和 36 kDa 的条带。像其他典型的蛇毒金属蛋白酶一样, Jerdonitin 优先降解人纤维蛋白原的 alpha 链, 并且该活性能被 EDTA 完全抑制, 而 PMSF 对其没有影响。Jerdonitin 不诱导小白鼠皮下出血。

关键词: 菜花烙铁头; 蛇毒金属蛋白酶; 出血; 纤溶酶活性

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Snake venom metalloproteinases (SVMPs) are found mainly in viper venoms. Most of SVMPs can

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* Corresponding author (通讯作者), E-mail: xiongyi@mail.kiz.ac.cn, chenrunqiang@126.com, Tel: +86–871–5192476,

Fax: +86–871–5196747

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cause hemorrhage due to the disruption of blood vessels and inhibition of platelet aggregation (Kamiguti et al, 1996).

According to their domain structures, SVMs are classified into four major groups (Bjarnason & Fox, 1994; Jia et al, 1996). Class P-I metalloproteinases (20–30 kDa) are composed of a single metalloproteinase domain with relatively weak hemorrhagic activity. Class P-II metalloproteinases (30–60 kDa) consist of a metalloproteinase domain and a disintegrin domain. Normally, these two domains will be separated by posttranslational processing and released separately. However, Jerdonitin from *Trimeresurus jerdonii* venom (Chen et al, 2003) and bilitoxin-1 from *Agkistrodon bilineatus* venom (Nikai et al, 2000) comprised metalloproteinase and disintegrin domains, which were classified as a new type of P-II class SVMs. Hemorrhagins of the class P-III are large toxins (60–100 kDa) with the most potent activity, and comprise multidomain enzymes built up by an N-terminal metalloproteinase domain and C-terminal disintegrin-like and cyteine-rich domains. Compared with the class P-III, class P-IV metalloproteinase contains an additional disulfide-linked C-type lectin-like domain (about 15 kDa).

In this study, we reported the purification and characterization of Jerdonitin, a new member of P-II class SVMs, which comprises metalloproteinase and disintegrin domains from *T. jerdonii* venom.

1 Materials and Methods

1.1 Materials

T. jerdonii crude venom was from the stock of the Kunming Institute of Zoology, the Chinese Academy of Sciences. Sephadex G-75 (superfine), Q-Sepharose high performance, Hi Trap Heparin HP and Resource Q columns were from Amersham Biosciences (Uppsala, Sweden). Ethylenediaminetetraacetic acid (EDTA) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Dongfeng Reagents Company (Shanghai, China). Low molecular weight markers, reagents for SDS-PAGE and human fibrinogen (plasminogen free) were purchased from Sigma (St Louis, MO, USA). Other reagents used were analytic grade.

1.2 Purification of Jerdonitin

1.2.1 Ion-exchange chromatography on Q-Sepharose high performance Lyophilized crude venom of *T. jerdonii* (1 g) was dissolved in 8 mL of 50 mM Tris-HCl buffer, pH 8.0. The solution was centrifuged and the supernatant was applied to Q-Sepharose high perfor-

mance column (100 mL), pre-equilibrated with the 25 mM Tris-HCl buffer, pH 8.0. After washing the column to remove the unbound materials, bound proteins were eluted with a linear gradient (0–0.32 M) of NaCl at flow rate of 240 mL/h and monitored at 280 and 215 nm on Pharmacia FPLC system (AKTA explore). Fractions containing the similar molecular weight with Jerdonitin and inhibition of ADP-induce platelet aggregation activity were pooled and lyophilized.

1.2.2 Gel filtration chromatography on Sephadex G-75 (superfine) The active fractions from the previous step were dissolved in 25 mM Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl, and centrifuged at $10\,000 \times g$ for 5 min. The supernatant was loaded on Sephadex G-75 (superfine) column (2.6×120 cm), pre-equilibrated with the same buffer. Flow rate was 9 mL/h and 3 mL fractions per tube were collected. The proteins were traced by monitoring the absorbance at 215 and 280 nm. Fractions containing the similar molecular weight with Jerdonitin and inhibition of ADP-induce platelet aggregation activity were pooled and dialyzed against 25 mM Tris-HCl buffer, pH 8.0 for the further purification.

1.2.3 Affinity chromatography on Hi Trap Heparin HP The active fractions from the prior step were applied to the Hi Trap Heparin HP column (5 mL), pre-equilibrated with 25 mM Tris-HCl buffer, pH 8.0. After washing the column to remove the unbound proteins, the bound proteins were eluted with a linear gradient (0–1 M) of NaCl at a flow rate of 60 mL/h and monitored at 280 and 215 nm on Pharmacia FPLC system (AKTA explore). Fractions containing the similar molecular weight with Jerdonitin and inhibition of ADP-induce platelet aggregation activity were pooled prior to the next step.

1.2.4 Ion-exchange chromatography on Resource Q

The active fractions from the preceding step were loaded to the Resource Q column (1 mL), pre-equilibrated with the 25 mM Tris-HCl buffer, pH 8.0. Elution was achieved with a linear NaCl gradient from 0 to 0.3 M in the same buffer at a flow rate of 0.5 mL/min and monitored at 280 and 215 nm on Pharmacia FPLC system (AKTA explore).

1.3 Determination of molecular weight

According to the method of Laemmli (1970), non-reducing and reducing (in the presence of 5% β -mercaptoethanol) SDS-PAGE were performed in 12.5% polyacrylamide gels. Molecular weight standards were rabbit phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (43.2 kDa), bovine

carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and hen egg white lysozyme (14.4 kDa). Protein was stained with Coomassie Brilliant Blue G-250.

1.4 Determination of hemorrhagic activity

Hemorrhagic activity was assessed by the modified method of Ownby et al (1978). Mice were injected subcutaneously with a sample dialysed against 50 μ L of 0.9% (w/v) NaCl solution. After 24 h, hemorrhagic spot was observed on the inner surface of the removed skin.

1.5 Fibrinogenolytic activity assays

Fibrinogenolytic activity was determined by a modified fibrinogenolytic assay (Gao et al, 1998). The fibrinogen solution (0.2 mL of 2% human fibrinogen in 50 mM Tris-HCl buffer, pH 7.6, 0.15 M NaCl) was mixed with 40 μ L of purified protein (10 μ g) and incubated at 37 $^{\circ}$ C for 0, 5, 15, 30, 60, 120 min and 5, 12, 24 h. After the indicated time intervals, an aliquot of 20 μ L reaction mixture was transferred to 20 μ L denaturing reagent (10 M urea, 4% SDS and 4% 2-mercaptoethanol) and incubated at 100 $^{\circ}$ C for 5 min. The sample was then analyzed by 12.5% SDS-PAGE.

1.6 Recalcification time assays

Recalcification time assay was carried out according to the method of Lee et al (1995). Briefly, normal citrated human plasma (100 μ L) was incubated at 37 $^{\circ}$ C for 1 h to achieve temperature equilibration. Then 100 μ L of sample was added and incubated at 37 $^{\circ}$ C for

2 min before plus 100 μ L of 30 mM CaCl_2 to initiate reaction. The clotting time was recorded. Accordingly, the pro-coagulant activity was expressed by shortening the plasma recalcification time while anti-coagulant activity by lengthening it.

2 Results

2.1 Purification of Jerdonitin

Following ion-exchange chromatography on Q-Sepharose high performance (Fig. 1A), gel filtration chromatography on Sephadex G-75 (superfine) (Fig. 1B), affinity chromatography on Hi Trap Heparin HP (Fig. 1C), and ion-exchange chromatography on Resource Q (Fig. 1D), a homogeneous Jerdonitin, as ascertained by SDS-PAGE, was obtained. From SDS-PAGE, it was a single chain with an apparent molecular weight of 36 kDa under reducing conditions and 38 kDa under non-reducing conditions, respectively (Fig. 2).

2.2 Hemorrhagic activity

Most of SVMPs cause hemorrhage. However, Jerdonitin (15 μ g) did not produce a hemorrhagic spot when injected intradermally in mice, indicating that Jerdonitin was a non-hemorrhagic SVMPs.

2.3 Fibrinogenolytic activity

It cleaved the α -chain completely and β -chain of fibrinogen after 24 h, leaving the γ -chain intact (Fig. 3).

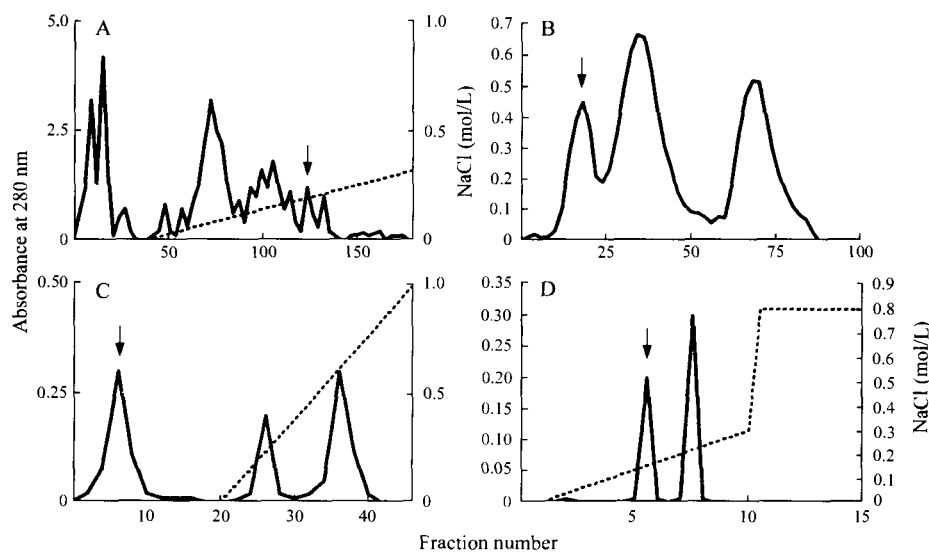


Fig. 1 Purification of Jerdonitin from *Trimeresurus jerdonii* venom

A: Ion-exchange chromatography on Q-Sepharose high performance column (100 mL); B: Gel filtration chromatography on G-75 (superfine); C: Affinity chromatography on Hi Trap Heparin HP column; D: Ion-exchange chromatography on Resource Q column. Bars represent Fractions containing Jerdonitin.



Fig. 2 SDS-PAGE analysis of Jerdonitin from *Trimeresurus jerdonii* venom

Lane 1: purified Jerdonitin (1.2 µg) under non-reducing conditions. The molecular weight was estimated to be 36 kDa. Lane 2: molecular weight markers. Lane 3: Jerdonitin (1.2 µg) under reducing conditions.

2.4 Recalcification time assay

The human plasma recalcification time of Jerdonitin was not changed, indicating that it had no pro-coagulant and anti-coagulant activities.

3 Discussion

Jerdonitin, a new type of P-II class snake venom metalloproteinase, comprises metalloproteinase and disintegrin domains. It has been isolated by gel filtration G-100 (superfine), Resource Q column (1 mL) and RP-HPLC C₄ column (Chen et al, 2003). However, no hemorrhagic and fibrinogenolytic activities for Jerdonitin were found. We think that organic buffer of HPLC might affect its enzyme activities. To study whether it has enzyme activity, we purified Jerdonitin by another procedure through a combination of ion-exchange chromatography on Q-Sepharose high performance (Fig. 1A), gel filtration chromatography on Sephadex G-75 (Superfine) (Fig. 1B), affinity chromatography on Hi Trap Heparin HP (5 mL) (Fig. 1C) and ion-exchange chromatography on Resource Q (1 mL) (Fig. 1D) columns. Homogeneous Jerdonitin, as ascertained by SDS-PAGE (Fig. 2), was obtained. It was a single chain with an apparent molecular weight of 36 kDa under reducing conditions and 38 kDa under non-reducing conditions, respectively.

Most of SVMPs cause hemorrhage. However, Jerdonitin (15 µg) did not produce a hemorrhagic spot when injected intradermally in mice, indicating that Jerdonitin was a non-hemorrhagic SVMPs. The relation of hemorrhage and protein primary structure has recent-

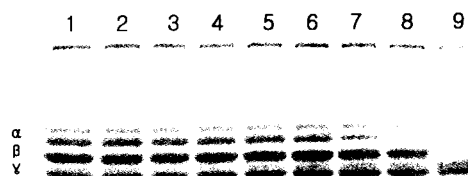


Fig. 3 Fibrinogen degradation by Jerdonitin: time-course analysis by 12.5% SDS-PAGE

Line 1: fibrinogen solution for 24 h; Lanes 2 - 9: Jerdonitin incubated with fibrinogen solution for 5, 15, 30, 60, 120 min and 5, 12, 24 h.

ly been studied (Ramos & Selistre-de-Araujo, 2004). The residue Asn183 (numbering in this paper, Fig. 4) was N-glycosylated in some hemorrhagic SVMPs and this post-translational modification could also influence the enzymatic specificity and the hemorrhagic potential. Moreover, elimination of the N-glycosylation decreased hemorrhagic activity of bilitoxin-1 by 75%, suggesting the importance of the N-linked glycosylation sites for hemorrhagic activity (Nikai et al, 2000). In our case, there was Asp instead of Asn in the 183 position, which may influence the hemorrhagic potential.

Compared with other P-II SVMPs, Jerdonitin has a typical primary structure comprising both metalloproteinase and disintegrin domains. Jerdonitin dose-dependently inhibited ADP-induced platelet aggregation with IC₅₀ of 120 nM though the RGD-containing disintegrin domain (Chen et al, 2003). Furthermore, Jerdonitin degraded fibrinogen through the metalloproteinase domain. It cleaved the α-chain completely and β-chain of fibrinogen after 24 h, leaving the γ-chain intact (Fig. 3). Its fibrinogenolytic activity could be completely inhibited by EDTA, but not by PMSF. The results indicated that Jerdonitin really has enzymatic activity. Most of the α-fibrinogenases, which specifically process the α-chain of fibrinogen, are metalloproteinases (Rodrigues et al, 2000). Jerdohagin was also an α-fibrinogenase purified from *T. jerdonii* (Chen et al, 2004). Analyzing their digestion time, we found that jerdohagin completely digested human fibrinogen α-chain in 5 h. However, Jerdonitin did not digest it completely in 5 h, indicating that Jerdonitin was a weak α-fibrinogenase.

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      10      20      30      40      50      60      70
Jerd -QQ----RYIELVIVADHRMYTKYDGDKEISSKIYETANNLNEIYRHLKIHVVLIGLEMWSSGELS KVTLS
Bili -ERYNP-YKYIELFLVDNRMVTKYNGDLKIKTRIYELVNIILNEIYRPLYIRVALVGIEFWCNKDLINVKSA
Brev -QRYNP-FRYVELVLVADKGMVTKNNGDLKIKTRMYELANNLNDIYRYMYIHVALVGVEIWSGDKITVT PN
HR1A QQRYLNNFRFIELVIVADYRMFTKFSNLNEVKTWVYEIVNTLNEIYRYLYVRVALVALEWVNSGDLSSVTLS
HR1B -QQRFPP-RRYIKLAIIVVDHGI VTKHGNLKKIRKWIYQLVNTINNIYRSLNILVALVYLEIWSKQNKITVQSA
Jara QQRYDP-YKYIEFFVVDQGT VTKNNGDLKIKARMYELANIVNEIFRYLYMHVALVGLEIWSNGDKITV KPD

      80      90      100      110      120      130      140
Jerd ADETLDSEFGWE RERDLLQRKRHDNAQLLTGMI FNEKIEGRAYKESMCDPKRSVGI VRDHRTRPHLVANRMAHE
Bili SSVTLASFANWRESVLPNRTSHDNAQLLTAIVFNRGVIGSAYPAGMCDPNRSVGTVDHSEINLQVAITMAHE
Brev VDDLSSFAEWRKTDLLTRKKHDNAQLLTAIDFNGPTIGYAYIASMCHPKRSVGIQDYSPINLVLSVMAHE
HR1A AYDTLDSEFGWE RKRDLKRKSHDNAQLLTAIDFNGTIIGLAHVASMCDPKCS TGVQDYSSRNLVAVIMAHE
HR1B SNVTLDLFGDWRESVLLKQRSHDCAQLLTTIDFDGPTIGKAYTASMCDPKRSVGIQDYSPINLVAVIMTHE
Jara VDYTLNSFAEWRKTDLLTRKKHDNAQLLTAIDFNGPTIGYAYIGSMCHPKRSVGIQDYSPINLVAVIMAHE

      150      160      170      180      190      200      210      *
Jerd LGHNLGFHHDGDSCTCGANSCIMSATVSNPSSRFSDCSLFQYSSDIHNPFTSRCLYNEPSKTDIVSFPVCG
Bili IGHNLGGMHGDNNSC TCGYSCIMLPALSDQPSKYFSNCSYIQYRDFIMNQ--DPQCILNEPSGTDIVSFPVCG
Brev MGHNLGIHHDGSGYCSG DYACIMGATISHEPSTFFSNCSYIQWDFIMDH--NPECVNEPLGTDIVSFPVCG
HR1A MGHNLGIRHDRENTCHANSCIMS AVISDQPSKYFSNCSHVQYWNYYINDD--EPQCILNEPLRTDIVSFPVCG
HR1B MGHNLGI PHDGNCTCGGFPCIMS PMISDPPSELFNSNCSKAYYQTF LTDH--KPQCILNAPSKTDIVSFPVCG
Jara MGHNLGIHHDGTSCSGDYPCIMGPTISNPEPSKFFSNCSYIQWDFIMNH--NPECVNEPLGTDII SFPVCG

      220      230      240      250      260      270      280      290
Jerd NYIMEVGEECD CGFPANCCQNPCCDAATCRLTPGSQCADGLCCDQCREMKGKTI CRIARGD-DLDDYCNGISAG
Bili NDILEVGEECD CGCPRNCQDPCCNAATCKKYSWVQCESGEECDQCRFKGAGTECRRAMGD-DPGGRCTGQSAD
Brev NELLEVGEECD CGTPENCQNECCDAATCKLKSQCGHGDCEQCKFSKSGTECRESMPECDPAEHCTGQSSE
HR1A NELLEVGEECD CGSPATCRYPCDDAATCKLHSWVECESGECCEQCRFRTAGTECRARRSECDIAESCTGHSAD
HR1B NELLEAGEECDCGSPENCQYQCCDAASCKLHSWVKCESGEECDQCRFRTAGTECRAAESECDI PESCTGQSAD
Jara NELLEVGEECD CGTPENCQNECCDAATCKLKSQCGHGDCEQCKFSKSGTECRASMSECDPAEHCTGQSSE

      300
Jerd CPRNFFHA
Bili CP-----RN
      310      320      330      340      350      360
Brev CPADV FHKNGQPCLDNYGYCYNGNCPIMYHQCYALWGADVDAEDSCFESNTKGNYYGYCRKENGKIPCAPE
HR1A CPTDRFHRNGQPCLNHG YCYNGNCPIMYHQCYALWGANATVAKDSCFEDNQKNDYGYCRKENGKIPCEPQ
HR1B CPTDRFHRNGQPCLNHG YCYNGKCPIMFYQCYFLFGSNATVAEDDCFNNNKKGDKYFYCRKENEKIPCAQE
Jara CPADV FHKNGQPCLDNYGYCYNGNCPIMYHOCYALFGADVYEAEDSCFKDNOKGNYYGYCRKENGKIPCAPE

      370      380      390      400      410      420
Brev DVKCGRLYCKDN SPGQNNPCKMFYSNEDEHKGMVLPGTKCGDGKVC SNHGCVDTVATAY
HR1A DVKCGRLYCSLG---NQLP CREFYFTPTDENIGMVDGTGKCGDKKVC SNRQCVDVNTAY
HR1B DVKCGRLFCDN---KKY PCHYNYS-EDLDFGMVDHGT KCADGKVC SNRQCVDVNEAYKSTTVFSLI
Jara DVKCGRLYCKDN SPGQNNPCKMFYSNDEHKGMVLPGTKCADGKVC SNHGCVDTVATAY

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Fig. 4 Comparison of the amino acid sequence of Jerdonitin with those of bilitoxin-1 and P-III class SVMPs which undergo autolysis

Jerd: Jerdonitin (Chen et al, 2003); Bili: bilitoxin-1 (Nikai et al, 2000); Brev: Brevilysin H6 (Fujimura et al, 2000); Jara: jarahagin (Paine et al, 1992). HR1a and HR1b (Kishimoto & Takahashi, 2002).

There are human prothrombin or Fator X activators in the snake venom, which had the pro-coagulant activity (Zhang et al, 1998a, b; Kini et al, 2001; Siigur et al, 2001; Samel et al, 2003). The human plasma recalcification time of Jerdonitin was not changed, indicating that it had no pro-coagulant and anti-coagulant activities. Recalcification time assay suggested that Jerdonitin did not effect on the human plasma coagulant factors. It should not be human prothrombin or Factor X activator.

In the P-III class SVMPs, there is a cysteine residue in position 218 (Fig. 4). It would form a disulfide bond with free cysteine in the succeeding disintegrin-like domain, and therefore the spacer and disintegrin domains can not be released from the metalloproteinase after the posttranslational processing (Fox &

Bjarnason, 1995; Siigur et al, 1996; Jeon & Kim, 1999). Cysteine 218 of Jerdonitin and bilitoxin-1, a new type of P-II class SVMPs, was very important for connect of their metalloproteinase and disintegrin domains.

Although the metalloproteinase and disintegrin-like domains of P-III class SVMPs will not be separated after the posttranslational processing, more and more evidence showed that these two domains would be cleaved by the autolysis (Fig. 5). Autolysis, adding to the molecular complexity of the venom, also plays a role in the biological complexity of the venom. Compared with bilitoxin-1 and P-III class SVMPs, which have autolysis, Jerdonitin had also the cysteine 218 in the spacer domain and its mature protein consisted of metalloproteinase and disintegrin domains. So Jer-

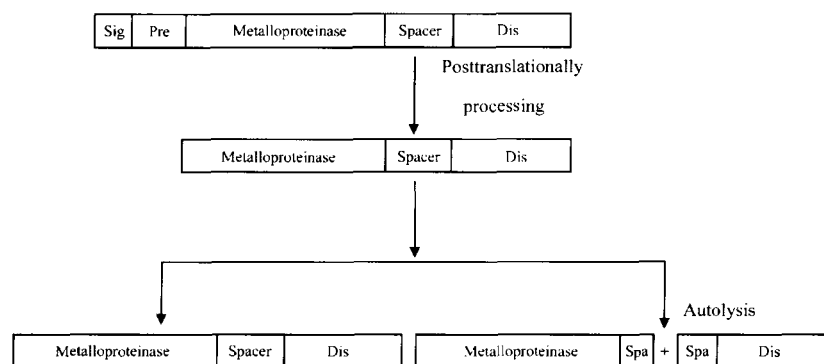


Fig. 5 Schematic models of the maturation process of SVMPs which undergo autolysis

Sig: signal peptide; Pre: prepeptide domain; Metalloproteinase: metalloproteinase domain; Spa: spacer domain; Dis: disintegrin domain or disintegrin-like and Cysteine-rich domains.

donitin might also undergo the autolysis.

In conclusion, we purified Jerdonitin in a new procedure from *T. jerdonii* venom. Jerdonitin cleaved the

α -chain of fibrinogen completely and β -chain partially. It is interesting that Jerdonitin didn't cause hemorrhage.

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